Genetic Diversity of Saltwater Crocodiles (*Crocodylus porosus*) from Sarawak Using Microsatellite Approach

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Declaration

I declare that this project entitled "Genetic Diversity of Saltwater Crocodiles (*Crocodylus porosus*) from Sarawak Using Microsatellite Approach" is the result of my own research except as cited in the references. The project has not been accepted for any degree and is not submitted in candidature of any other degree.

Signature :

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Date : 23rd Mei 2011

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List of Abbreviations

DNA	Deoxyribonucleic acid
T	Thymine
A	Adenine
G	Guanine
C	Cytosine
SSRs	Simple Sequence Repeats
PCR	Polymerase Chain Reaction
CIA	Chloroform- isoamyl alcohol
CTAB	Cetyl trimethyl ammonium bromide
µL	Microliter
mL	Milliliter
Rpm	Rounds per minute
OD	Optical density
UV	Ultra-violet
RNA	Ribonucleic acid
mM	Millimole
TAE	Tris-acetate electrophoresis
Bp	Base pair
Kb	Kilobase
Ng	Nanogram

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ABSTRACT

Crocodylus porosus (saltwater crocodile) locally known as Bujang Senang is the common crocodile found in Sarawak. There were several genetic studies related to *C. porosus* in Sarawak that have been conducted, however relationships between *C. porosus* in Sarawak remained unresolved. Therefore, this study is aimed to assess genetic diversity of *C. porosus* obtained from different locations in Sarawak using microsatellite approach. Available *C. porosus* tissues samples were subjected to total genomic DNA extraction using modified CTAB method, Polymerase Chain Reaction – Simple Sequence Repeats (PCR-SSRs) and data analysis. Microsatellite loci for *C. porosus* from Sibu, Miri and Bako of approximately 115 to 122 base pairs (bp) were successfully obtained during this study. *C. porosus* from Sibu showed repeat motifs of $(GT)_{22}$ $(GA)_5$, Miri $(GT)_{21}$ $(GA)_5$ and Bako $(GT)_{20}$ $(GA)_5$. Phylogenetic tree construction using Unweighted Pair Group with Arithmetic Averages (UPGMA) and Maximum Parsimony (MP) methods revealed monophyletic grouping of *C. porosus*.

Keywords: saltwater crocodiles, genetic diversity, microsatellite, repeat motif and monophyletic

ABSTRAK

Crocodylus porosus (Bujang Senang) merupakan spesies buaya yang biasa dijumpai di Sarawak. Beberapa kajian berkaitan C. porosus di Sarawak telah dijalankan namun hubungkait antara setiap individu dalam spesies itu masih menjadi persoalan. Oleh sebab itu, penyelidikan ini diadakan bertujuan untuk menilai kepelbagaian genetik C. porosus dari lokasi yang berbeza di Sarawak dengan menggunakan pendekatan mikrosatelit. DNA telah diekstrak daripada sampel C. porosus yang telah sedia ada dengan menggunakan kaedah CTAB yang diubah suai, diikuti dengan Polymerase Chain Reaction – Simple Sequence Repeats (PCR-SSRs) dan analisa data. Kajian ini telah berjaya mendapatkan lokus mikrosatelit untuk C. porosus dari Sibu, Miri dan Bako dengan panjang nukleotida 115 hingga 122 bp. C. porosus dari Sibu menunjukkan pengulangan motif $(GT)_{22}$ $(GA)_5$, *Miri* $(GT)_{21}$ $(GA)_5$ *dan Bako* $(GT)_{20}$ $(GA)_5$. *Analisa susur-galur menggunakn kaedah Unweighted Pair Group with Arithmetic Averages (UPGMA) dan Maximum Parsimony (MP) menunjukkan pengkelasan monofiletik untuk C. porosus dari ketiga-tiga tempat itu.*

Kata kunci: Bujang Senang, kepelbagaian genetik, mikrosatelit, pengulangan motif, monofiletik

1.0 Introduction

Crocodylus porosus (saltwater crocodile) is the largest reptile that fall under order Crocodylia and genus *Crocodylus*. Crocodylia is a small order within the class Reptilia. About 23 species under this order belong to eight genera. The largest genus is represented by the genus *Crocodylus* which consists of 11 species (Meganathan *et al*., 2010). Most of phylogenetic analyses were conducted using this genus to establish the basic structure of the crocodilian phylogeny. Genetic analysis on the saltwater crocodile, most broadly distributed crocodilian species also has been conducted due to it special conservation and economic interest.

C. porosus are found from Sri Lanka and the east coast of India in the west to the Caroline Islands in the east and from Myanmar and south-east Asia in the north to Australia in the south. This species inhabit coastal rivers and swamps, open sea and island shorelines. However, their distribution extends more to inland areas from the major rivers and floodplain into freshwater rivers, creeks and swamps. High density of this species also could be found in the tidal portions of some mangrove-lined rivers; predominantly those associated with extensive freshwater wetlands or floodplains. Therefore this saltwater crocodile also may occur in any salt or freshwater within their range (Leach *et al*., 2009).

Saltwater crocodile usually breed during the wet season. Females' crocodile will lay their eggs in a mound of grasses or other vegetation close to permanent water. The most frequently used nesting habitats are freshwater swamps close to tidal rivers and saltmarsh

habitats (Leach *et al*., 2009). Leach *et al*. (2009) also described that in Australia, high nesting effort is significantly influenced by years with high rainfall and cool conditions between August and November. Conversely, years with poor rainfall and hot conditions between August and November are related with low nesting effort. The number of eggs laid is proportional to the size of invidual female. Therefore larger female will produce large number of eggs. During the nesting, some of the eggs laid will be infertile and have high mortality rate. Flooding is major factor that caused the eggs to be damaged.

As a part of consumers in trophic level, crocodile plays an important role to balance structure and function of their habitat mainly in aquatic ecosystem. It acts as a predator that consumed on aquatic fauna thus help in control population of that species in the water bodies. Other than that, it also indirectly helps in maintaining water quality by feed on death animals found in the water. According to Leach *et al*. (2009), economical value of crocodile skins, flesh and body parts have increased income level for human. However, overpopulation of this species will cause danger to human livelihood.

Preliminary survey of the crocodile resource in Sarawak has been conducted by Cox & Gombek (1985). In the study, morphological characters identification has been used to document common crocodile species that exist in Sarawak. In addition, crocodile night counts also have been conducted in order to determine their distribution. A preliminary molecular study on *C. porosus* in Sarawak also has been conducted by Koh Hui Eng (2008) but number of samples used in her study was limited. Nur Sara *et al*. (2010) used Cyt *b* (Cytochrome-*b*) and 12S rRNA (ribosomal ribonucleic acid) gene to study the genetic diversity of this species but the relationships between *C. porosus* in Sarawak are

still unresolved. Therefore, this study is aimed to assess genetic diversity of *C. porosus* obtained from different locations in Sarawak using microsatellite approach and to determine molecular marker for that species since there is lack of information on the molecular study and population structure of *C. porosus* in Sarawak. It is hoped that the microsatellite data will be used for the future study, particularly in managing the crocodile-human conflict in Sarawak.

2.0 Literature Review

2.1 Taxonomical Review

The genus *Crocodylus* inhabiting tropical areas and comprises of 23 named species (commonly referred to as the true crocodiles) that range from the largest living reptile and largely distributed *C. porosus*, to small-bodied, narrowly distributed island endemics such as *C. novaeguineae*, *C. mindorensis*, and *C. rhombifer* (Oaks, 2007). Table 2.1 showed the taxonomy of *C. porosus*.

Table 2.1: Taxonomy of *C. porosus* (Oaks, 2007).

Kingdom: Animalia
Phylum: Chordata
Class: Reptilia
Order: Crocodylia
Family: Crocodylidae
Genus: *Crocodylus*
Species: *Crocodylus porosus*

C. porosus possess pores in their skull which are functions in reducing weight of the skull without lowering its power. Blood vessels located in their sensory bumps help in detecting changes in water pressure. This is one of their special ability to detect the presence of their swimming prey. Its common English name is saltwater crocodile or

estuarine crocodile and locally known as ‘Buaya Tembaga’. It is also known as ‘Buaya Muara’ in Indonesia. In Sarawak, it is sometimes referred as 'Bujang Senang' or 'happy bachelor' (Sarawak Forestry, 2010).

2.2 Economical Value of Crocodiles

In Sarawak, overexploitation on *C. porosus* occurs when local people started to trade the crocodile’s meat, gallbladders, wet salted skin, hatchlings, yearlings and skin export (Cox & Gombex, 1985). In Northern Territory (NT) Australia, saltwater crocodile is harvested for their skins, flesh and body parts. The reptile also is harvested ranges from their eggs until adults. The NT position in the world market for farmed crocodile skins is small but occupies an important and significant role due to its ability to supply high quality grade skins for good market fashion accessories.

Between 2003 and 2007, NT exported on average approximately 6,000 skins per year throughout the state and world countries. Developing farm infrastructure and increasing in the number of crocodiles reared indicates that this number will increase significantly. Although farming industry is not a big business, it is substantial in economic production with yearly turnover in the order of several tens of millions of Australian dollars. Six functional crocodile farms in the NT have created job opportunities for about 60 to 100 people (Leach *et al.*, 2009).

Wild and farm crocodiles contribute to the number of tourists visiting Australia. In visitor surveys done by the previous researcher, Leach *et al.* (2009) reported that seeing

crocodiles dominates the best experiences in wildlife-viewing. Even though tourists generally prefer to observe crocodiles in the wild, captive crocodiles are also rated highly and one of the popular place to be visited.

2.3 Threats to Crocodiles

Anthropogenic factors are the major factor that significantly impacts all crocodilian either directly or indirectly. Public demands for more strong crocodile management in areas close to human residential area also will result in the localized removal of increased numbers of animals (Leach *et al*., 2009). Number of adult saltwater crocodile also has been reduced through predation from other larger crocodile while young hatchlings crocodile are consumed by other animals such as fish and birds.

C. porosus populations also could be influenced by drought but the impact is for a while unless it is together with other factors. Heavy rainfall and subsequent flooding can cause damage to egg and death of juvenile (Leach *et al*., 2009). Sea level rises will cause damage to crocodile habitat mainly their nesting site. Population structure also could be affected since their sex of hatchlings is influenced by the temperature. Other than that, shortage of food sources and low survival rate could be worsen if extreme dry season and storm events occur.

Quite a large number of crocodiles in Australia have been killed due to fishing traps. This incident is rarely happen in Malaysia. Recorded and assessment on the losses of *C. porosus* which has been caused by accidental capture and drowning in barramundi fishing

nets has been conducted in the early 1980s (Leach *et al*., 2009). Based on the information, commercial fishing has been prohibited within a number of river systems that are important as nesting habitats for *C. porosus,* such as the Mary, Roper and Alligator Rivers. Fishermen also are not permitted to use wild crocodiles that drown in their nets.

2.4 Conservation Status on *C. porosus*

Over the past three decades, *ex-situ* approach and managed-harvest of crocodilian have been held up as a success story in the search for balanced, sustainable use of wildlife and the generation of wildlife products for international trade (Thorbjarnarson, 1999). The success of the managed-harvest programs in the United States, Zimbabwe, Papua New Guinea, and Venezuela encouraged similar efforts in a variety of other countries including Malaysia.

Programmes for the conservation of crocodiles, alligators and caimans collectively known as crocodilians have been designed in most countries due to the high demand of products from the consumptive use of wild animals. Some of these schemes have successfully contributed to the extensive conservation benefits. However, there are also some difficulties and failures that exist during the management programmes, which are more infrequently documented. Although most crocodilian production programmes started with strong conservation objectives, it has often been difficult to hold on these over the long term. Therefore, good relationship and strong correlation between government regulators, private sectors and business interests, from the planning stages forward are needed to overcome these problems.

C. porosus is listed in Appendix I in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2009) in all countries which means that necessity trade is very restricted. However, it is listed in Appendix II in Australia. Environment Protection and Biodiversity Conservation (EPBC) Act and Territory Parks and Wildlife Conservation (Northern Territory) Act in Australia also listed it as protected species but not listed as threatened (Leach *et al*., 2009). It is also listed under The Protection of Wildlife Act 1972 (Act 76) as a protected species in Malaysia and Wild Life Protection Ordinance 1998 in Sarawak. In contrast, International Union for the Conservation of Nature (IUCN) Red List of Threatened Species has listed them as least concern on the 2009 (Nazli *et al*., 2009).

2.5 Genetic Diversity

Biodiversity is defined based on three important concepts in biological organization level namely genetic, species and community. Hughes *et al*. (2008) defined genetic diversity as any marker that can reveal the magnitude of genetic variability within a population. Genetic diversity is important to determine the biodiversity level of a species or population. An evolution and adaptation of the population to environment changes are not possible without influenced from genetic diversity. Other than that, it also reveals major impacts on ecological processes such as primary productivity, population recovery from disturbance, interspecific competition, community structure, fluxes of energy and nutrients (Hughes *et al*., 2008). This is because the survival of species is depends on the stable

genetic variability within and among population. The genetic variation has enabled that population to adapt in new environment which is usually created by natural phenomena and anthropogenic factors.

Previously, morphological study of the flora and fauna has been the most widely used technique for a long time. However, morphological characteristics are often restricted because not all characters of the species can be obvious at all stages of their life cycle. Sometimes, this appearance could be affected by environmental factors. Nowadays, a variety of different genetic markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified polymorphic length polymorphism (AFLP), and microsatellite or simple sequence repeats (SSRs) have been proposed to assess genetic variability in genetic resources management (Powell *et al*., 1996). These molecular techniques are more advanced since they can provide valuable data on diversity through their ability to detect variation at the DNA level which is significant for the conservation purpose. The importance of genetic diversity in ecological is stated by Zhang *et al*. (2002) which said that species with small genetic variations may be more vulnerable to environment changes and has high risks to be endangered.

2.6 Microsatellite

Microsatellite is simple sequence repeats (SSRs) that consists of 1 to six nucleotides repeats (Holton, 2001). They are found in a wide variety of eukaryotes and also in the genome of plant's chloroplast (Jarne & Lagoda, 1996). After it has been emerged as the

PCR- based genetic marker, this molecular tool has been most frequently used technique in order to address question about genetic diversity and population study in a different type of living organism. Microsatellites are common in most eukaryote genomes and provide hyper-variability in the single locus marker which means that it is effective in estimating migration and relatedness among individuals.

The most widely used types of microsatellite are dinucleotide repeats, trinucleotide repeats and tetranucleotide repeats with the density of each microsatellite varies widely within the same and different species (Jarne & Lagoda, 1996). Dinucleotide is repeating of two base pairs in the DNA sequence such as CA repeats which are dominants in Animal Kingdom. TA or GA repeats are rich in plants however polymorphism might be lower than animals (Jarne & Lagoda, 1996). Trinucleotide is largely studied since it advantages human in terms of medicinal value specifically in diseases and cancers. The example is GTG repeats which preferentially localized on human chromosomes (Jarne & Lagoda, 1996). Tetranucleotide repeats of GATA and GACA mostly found in higher organisms and highly polymorphic. Nevertheless, this type of microsatellite repeats is not widely used in population biology studies.

2.7 Molecular Research (Microsatellite) on Crocodilians

Microsatellite markers have been isolated for a number of crocodilian species including *Alligator mississipiensis*, *Crocodylus moreletii*, *Crocodylus johnstoni*, *Caiman latirostris*, and *Crocodylus porosus*. Some of these markers have successfully amplified in *Caiman*

crocodiles (Oliviera *et al*., 2010). However, levels of polymorphism are often low, and microsatellite patterns can be difficult to interpret in *C. crocodilus*, making heterologous markers inefficient for analyses of mating systems and fine-scale population structuring. Therefore, a study on microsatellite markers for mating system and population analyses of the spectacled caiman *C. crocodiles* have been conducted (Oliviera *et al*., 2010). In this study, 12 dinucleotide microsatellite loci of the *C. crocodiles* have been isolated and characterized using microsatellite enriched library. It is about 21 individuals of caiman *C. yacare* from the different place in the Brazil have been used as the subspecies in order to compare the result. Results showed that there are some differences in their genetic structure with number of alleles varying from three to 20 and one to 14 per locus in both species. Although 12 polymorphic loci had similar characteristics in the caiman *C. yacare*, only 10 loci were polymorphic (Oliviera *et al*., 2010). Oliviera *et al*. (2010) concluded that characterized locus is significant for further evolutionary analyses of the *C. crocodiles* species.

Miles *et al*. (2009a) isolated and characterized 253 novel and polymorphic microsatellite loci for the *C. porosus* by constructing libraries enriched for microsatellite DNA. This is due to the requirement of large genetic resources to conduct the study on phylogeny or population structure of new organism through molecular study. The data is also important for the future use in the construction of a genetic-linkage map for the saltwater crocodile. The isolation, primer development, and polymerase chain reaction (PCR) conditions to amplify a total of 253 novel and informative microsatellite from studied species are well described in their publication. In the study, 421 loci are successfully amplified with 253

microsatellite loci have been identified as polymorphic which means that high number of alleles were detected and this shows that SSRs is an informative marker (Miles *et al*., 2009a).

According to Miles *et al.* (2009a), higher cost for the microsatellite DNA isolation and development, labor intensive and lack of suitable universal primers which has the ability to amplify the homologous loci in a wide range of species are some of the limiting factor that affect the effectiveness of application of microsatellite method in crocodilian research, especially for "true crocodiles". It was supported by Holton (2001) in his statement that said isolation of useful microsatellite loci can be time consuming and expensive. Therefore, a research on the cross-species amplification of 82 existing microsatellites previously isolated for the saltwater crocodile (*C. porosus*) has been conducted in 18 non-target crocodilian species. It is done by the selection of 82 microsatellites for whole genome scans based on their relatively even map distribution and high polymorphic content in the *C. porosus* mapping resource from the 253 novel microsatellites developed by Miles *et al*. (2009b). The markers chosen were those displaying the highest levels of polymorphism in *C. porosus* which are significantly important for the genetic markers to be apply in population and evolutionary genetic studies. Present investigation was able to observe high level of cross-amplification among true crocodilians since the data obtained from the study have provided many polymorphic microsatellites for a range of Crocodylia species which is previously has less information on it genetic markers (Miles *et al.*, 2009b).

3.0 Materials and Methods

This section is divided into 2 sub-sections which are laboratory work and data analysis.

3.1 Laboratory Work

This section consists of total genomic DNA extraction, agarose gel electrophoresis, optical density reading, Polymerase Chain Reaction – Simple Sequence Repeats (PCR –SSRs), PCR products purification and sequencing.

3.1.1 Total Genomic DNA Extraction (Adapted from Grewe *et al*., 1993)

Approximately 1-2 mm of *C. porosus* tissue samples from 5 locations in Sarawak were minced. Those tissue samples were collected by previous researcher from Sibu, Miri, Serian, Kuching (Bako 1) and Kuching (Bako 2). Details of samples used in this study were listed in Table 3.1.

Table 3.1: Available samples of *C. porosus* which involved in this study.

No.	Voucher No.	Location	Type	Notes
1	SN 001	Serian	Tissue	Hard tissue
2	MR 001	Miri	Tissue	Soft tissue
3	SB 001	Sibu	Tissue	Soft tissue
4	BK 002	Kuching (Bako 1)	Tissue	Soft tissue
5	BK 003	Kuching (Bako 2)	Tissue	Soft tissue and foul smell

Minced samples were placed into different 1.5 mL microcentrifuge tubes which contain 700 µL of 2X cetyl-trimethyl ammonium bromide (CTAB) buffer. Then, 7 µL of Proteinase K was added into the tubes. After that, it was incubated in the water bath (PROTECH, Model-903) at 60 ºC. The tubes were incubated until the sample dissolved completely which it was about 1 hour 30 minutes. After that, 700 µL of chloroform-isoamyl alcohol (CIA) was added into the tube, followed by gentle shake for 1-2 minutes to mix the solution. The tubes were centrifuged at 13000 rounds per minute (rpm) for 10 minutes with 4°C in high-speed micro centrifuge machine (HITACHI RX series, Model-CF 15RX). Three layers of mixture were observed in the tubes. An amount of 600 µL upper layer aqueous phase was taken out slowly from the tube by using micropipette and transferred into a new tube. An equal amount of 100% ethanol (EtOH) was added into each tube. The tubes were inversed slowly to make sure that the mixture was properly mixed. It was stored overnight in the -20°C freezer. Next, it was centrifuged at 13000 rpm for another 10 minutes with the same temperature used. The excess ethanol was removed and 500 µL of 100% ethanol, EtOH, was added into the tube. Later, 25 µL of 3M sodium chloride, NaCl, solution was added into the tube and the mixture was mixed. The tubes were centrifuged again at 13000 rpm for 10 minutes. Excess ethanol was removed completely. The pellet was ensured to be at the bottom of the tube. The tubes were left on the bench work at room temperature to dry the pellet. Finally, the pellet was dissolved in 50 µL sterilized distilled water (ddH_2O). DNA extraction products were stored in -20 ºC freezer for future used.

3.1.2 Agarose Gel Electrophoresis

Electrophoresis using agarose or polyacrylamide gels is the standard method used to separate, identify, and purify DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation (Sambrook *et al*., 1989). Although agarose gel has lower resolution power than polycarylamide gel, it has a greater range of separation. Various concentrations of agarose gels are able to separate DNA fragments from 200 bp to approximately 50 kb in length.

In this experimentation, a total of 0.5g agarose powder was weighted by using an analytical balance and placed in a 250 ml non contaminated beaker. Then, 50 ml of 1x tris-acetate electrophoresis (TAE) buffer was poured into the same beaker in order to prepare 1 % agarose gel. Agarose powder with 2 g in weight was mixed with 100 ml of 1x TAE for the preparation of 2 % agarose gel. Beaker was placed in the automatic microwave for 2 minutes to melt the agarose gel. After 2 minutes, the beaker was taken out from the automatic microwave and the melting agarose gel was poured into a contaminated beaker. Approximately 1µl of ethidium bromide was added into the gel solution in the contaminated beaker. The beaker was swirled to mix the solution and poured into the prepared tray. Comb was placed inside the tray to form wells. The solution was allowed to solidify. After that, comb was removed from the solidified gel. The 1% agarose gel then was placed in the prepared gel electrophoresis tank that contained 1x TAE buffer with the wells samples at the negative terminal. Mixture of 2µl 1kb DNA ladder and 2 µl 6X loading dye were expelled into the first wells, while other wells were filled with the